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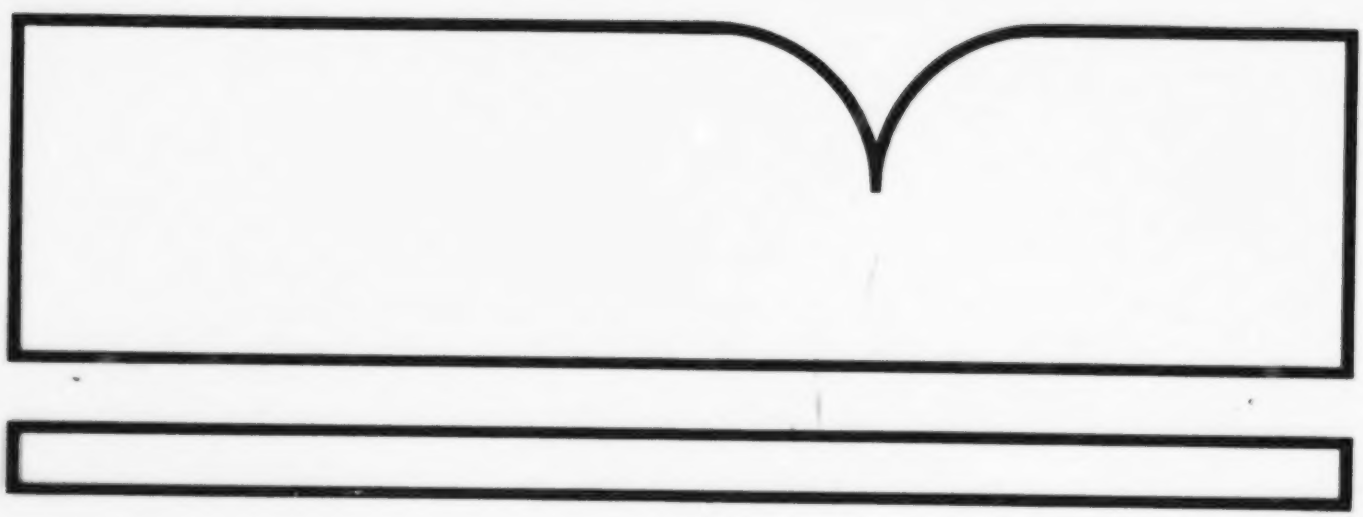

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16. ABSTRACT

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CHAPTER 47

Chitin Transformation and Pesticide Interactions in a Simulated Aquatic Microenvironmental System

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Interactions between the structural aminopolysaccharide, chitin, and the organophosphate pesticide, azinphosmethyl (Guthion®), have been studied in a controlled continuous flow-through microcosm. Pesticide-induced microbial population changes and increases in substrate utilization of chitin are noted. Significant increases in total heterotrophs and chitinoclasts occurred relative to continuous application of azinphosmethyl. Rapid uptake and utilization of chitin and its derivatives, enhanced by controlled azinphosmethyl additions, were examined using a tagged chitin breakdown product, N-(glucosamine-1-^{14}C)-acetyl-d-glucosamine. Rates of utilization coincided with rapid $^{14}CO_2$ release, assimilation of ^{14}C by microbial cells, and increases in colony-forming units. Data correlations, using microbial and enzymatic criteria, indicate the application of the benchtop microcosm in analyses of compound impact and substrate turnover.

INTRODUCTION

Along with cellulose, the structural polysaccharide chitin, a polymer of N-acetyl-d-glucosamine, is among the most abundant of carbon substrates in aquatic ecosystems. Turnover of this biodegradable material is critical in the recycling of carbon and nitrogen in aquatic environments and is a process mediated by a variety of chitinoclastic organisms. Earlier studies (Seki 1965; Chan 1970; Hood 1973) have demonstrated the relative abundance of chitin and chitinoclasts in estuarine environments. More recent work (Hood and Meyers 1977a,b) has shown correlations between chitinolytic processes and the biology of commercially valuable marine shrimp, *Penaeus setiferus*, and have indicated noteworthy levels of chitin transformation in salt-marsh environments of southeast Louisiana. The manifold aspects of chitin and the complexity of its contribution to aquatic production are noted in a recent symposium on the polymer and its derivative chitosan (Muzzarelli and Pariser 1978).

In conjunction with our studies on the impact of selected xenobiotics on microbial ecology and substrate turnover, efforts have been directed toward development of analytical predictive mechanisms to discern problems between the maintenance of wetland productivity and chemical discharges from adjacent agricultural and industrial areas. The vast wetland area of southeast Louisiana comprises a variety of ecologically diverse regions in which economically significant agricultural activities occur adjacent to highly productive brackish nursery grounds that serve the largest commercial fisheries in the United States. The pesticide studied was Guthion ® (azinphosmethyl), an organophosphorus compound representing approximately 80% of the total volume of insecticides applied to sugarcane to control the cane borer. Guthion was substituted for endrin 10-12 yr ago because of problems with the latter in toxicity to fish and resistance to insects.

The mechanism used comprised a microcosm, or microenvironmental system, to stimulate the target field environment under controllable laboratory conditions. Such microcosms provide a correlated interpretative approach to field studies, thus contributing needed data on fate and transport of short-lived compounds of biological significance. The application of the microcosm approach in assessing the effect of toxic chemicals is well documented by Hague (1980) and has been discussed by other investigators (Pritchard et al. 1979; Portier 1979). The work reported here involves studies of chitin turnover and the impact of Guthion and a variety of biotic and abiotic factors on this process.

MATERIALS AND METHODS

Sample Collection

Soil and sediment collections were made in the Lac des Allemands environment of the drainage basin of Barataria Bay, Louisiana. This watershed region comprises distinctive vegetative zones and areas of contrasting salinity, all affected by a gulfward movement of water. The brackish and freshwater regions, located adjacent to intensively cultivated agricultural fields, are affected most directly by runoff.

Cores consisting of aluminum cylinders, 60 cm tall x 7.6 cm diameter, were inserted into the sediment to the water level, capped and sealed, and stored on ice for transportation to the laboratory for microbial analysis. In drier sites, an inclined trench to a depth of 60 cm was dug, with an undisturbed wall at one end for asentic sampling. All samples were processed within a 12-h period. Sediment from the particular sites was used to inoculate individual microcosms and to categorize the major microbial groups present.

Enumeration of Microorganisms

For enumeration of total heterotrophic aerobes and anaerobes, sediment samples were diluted appropriately with phosphate buffer, pH 7.2. Using the pour-plate method, replicate 0.1-ml samples were added to standard Plate Count Agar (Difco), incubated for 3 d at 26 C, followed by enumeration of colony-forming units (CFU). For anaerobic studies, proper atmospheric conditions were maintained by using a disposable hydrogen/carbon dioxide envelope (GasPak ®)(BBL) in the presence of a cold

catalyst in an anaerobic jar. A methylene blue indicator strip was used to insure anaerobiosis. Plates were incubated at 36 C and examined every 24 h.

Four groups of microorganisms, i.e., bacteria, actinomycetes, filamentous fungi, and yeasts, were enumerated in terms of biomass present based on CFU. Appropriate replicate dilutions were made of all sediment samples using the pour-plate method. For enumeration of bacteria and actinomycetes, replicate 1.0-ml aliquots were added to Jensen's agar medium (Jensen 1930). Cycloheximide (Sigma), an antifungal agent, was added at 40 µg/ml to inhibit overgrowth by filamentous fungi. For enumeration of filamentous fungi and yeasts, Martin's agar medium (Martin 1950) was used together with streptomycin (Sigma) and chlortetracycline (Sigma) at 30 µg/ml to retard bacterial growth. All plates were incubated at 30 C and examined after 3 d. Colonies were enumerated using a scan dissection microscope to distinguish the four major morphological groups.

Chitinoclasts were enumerated using a chitin medium containing 2.5% precipated chitin (Sigma) plus mineral salts (Hsu and Lockwood 1975). Preparation of the precipated chitin followed the methods of Okutani (1966) and Hood (1973). Pure chitin, ball-milled for 72 h at 4 C, was dissolved in concentrated HCl in quantities of 10 g/150 ml. The dissolved chitin was added to a large vol of distilled water. The milky white precipate was washed repeatedly to remove acid, and the solution was adjusted to give a value of 10 mg/ml at pH 7.2. The plates incubated at 30 C were inspected after 3 d. Colonies exhibiting clearing zones were diagnostic of chitin utilization.

Enzyme Assay

Chitinase. The assay for chitinase activity followed the method described by Okutani (1966) using the N-acetyl-d-glucosamine assay (Reissig et al. 1955). A crude enzyme extract was obtained by filtering 10 ml of culture broth aseptically collected through a sterile 0.45 µ cellulose acetate filter. One milliliter of cell-free liquid was added to an enzyme assay solution (Hood 1973), incubated for 7 d at 30 C, and N-acetyl-d-glucosamine determined spectrophotometrically at 585 nm.

Phosphatase. Phosphatase activity was determined using the procedure outlined by Tabatabai and Bremner (1969) and modified by Atlas et al. 1977). Replicate 2-g aliquots of control and pesticide-treated sediment were placed in 150-ml Erlenmeyer flasks with 0.1 ml of toluene, 4 ml of sterile distilled deionized water, and 0.5 mg/ml of p-nitrophenol phosphate (Sigma). Flasks were incubated at 30 C for 100 min. With the addition of 1 ml of 0.05 *M* calcium chloride, 4 ml of 0.5 *M* sodium hydroxide, and 20 ml of distilled deionized water, each sample was centrifuged at 10,000 x *g* for 10 min and the resulting yellow solution assayed at 410 nm for p-nitrophenol (PNP) production.

Dehydrogenase. Dehydrogenase determination was based on formation of 2,3,5 triphenyltetrazolium formazan (TPF) from a standard of 2,3,5 triphenyltetrazolium chloride (TTC). Replicate 10-gm aliquots of control and pesticide-treated soils were placed in 250-ml Erlenmeyer flasks and brought to 100% water-holding capacity with addition of a

0.5% solution of 2,3,5-triphenyltetrazolium chloride (Sigma), as described by Bartha et al. (1967). Each flask was flushed with O_2-free nitrogen, tightly stoppered, and incubated for 6 h (Casida 1977). The TTC is converted by dehydrogenase enzymes to TPF, forming a characteristic red color. The latter was extracted with 50 ml of spectroscopic grade methanol (Baker Chemical), centrifuged, and filtered through No. 5 filter paper (Whatman). Absorption was measured at 485 nm and dehydrogenase activity units expressed in mg/ml TPF.

Continuous Flow Microcosm Studies

The microcosm system used (Portier 1979) is shown in Fig. 1A. The design of this bench-top microcosm allows introduction of a variety of analytical features including: variation in flow rate and sequential compound addition; control of pH/Eh, temperature, O_2, and salinity; maintenance of a soil/water interface; and generation of controlled quantitative data. Furthermore, the entire apparatus is autoclavable for aseptic operations and is capable of maintaining the desired parameters of temperature, flow rate, and pH for a period of 5 wk or longer.

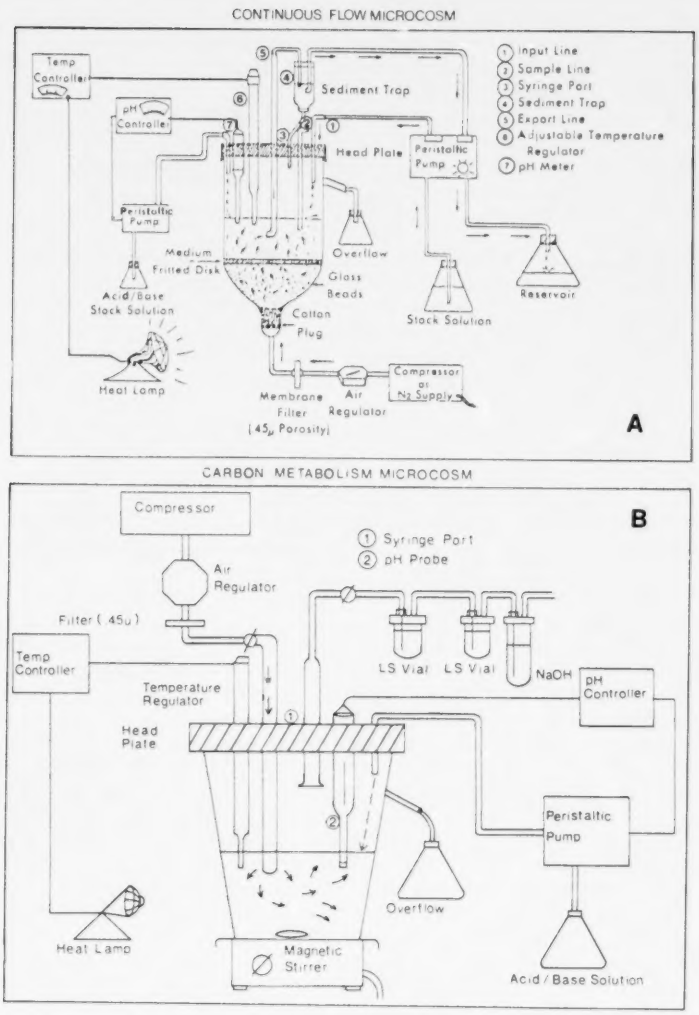
For the controlled microcosm studies, sediment from the Lac des Allemands site was placed into four continuous flow-through microcosms. Approximately 50 g (wet wt) of sediment was introduced with appropriate amounts of filter-sterilized (0.45 μ porosity cellulose acetate filter) site water to give a final vol of 1 L. Two microcosms served as controls while a 10-ppm solution of the pesticide azinphosmethyl was introduced into the two test microcosms following equilibration. The test solution, added at a flow rate of 0.105 ml/min, consisted of filter-sterilized site water, followed subsequently by site water amended with azinphosmethyl for test microcosms. Temperature was maintained at 29 C, with the pH at 7.2. Temperature and pH control was maintained effectively using a Versa Therm Model No. 2158 and Horizon Model No. 5997, respectively.

Carbon Metabolism Microcosm Studies

A static microcosm (Fig. 1B) was designed for studies involving analyses of chitin breakdown rates by production of N-acetylamino sugars in chitin-supplemented reaction chambers. The substrate used was ^{14}C-labeled N-acetyl-d-glucosamine (NAG), with subsequent analyses of $^{14}CO_2$ production via liquid scintillation techniques.

Sediment from the field site was dispensed in 25-g aliquots into four static microcosms with 400 ml of sterile distilled deionized water. A labeled solution and a nonlabeled aliquot of NAG was added to each microcosm to give a final concentration of 0.1071 μCi/500 ml. The specific activity of labeled NAG was 58.18 mCi/mmol. The final concentration of both labeled and nonlabeled NAG was 40 ppm. Temperature of the vessels was maintained at 28 C, with a pH range of 6.8-7.2. Two microcosms served as controls, while the test chambers included a final pesticide concentration of 10 ppm Guthion.

A moderate airflow into each microcosm allowed for entrapment of $^{14}CO_2$ by liquid scintillation vials containing 15 ml of complete oxidizer cocktail (New England Nuclear). To determine uptake rates of labeled compound, replicate 1-ml aliquots from 10^5 sample dilutions



1. (A) Diagram of continuous flow microcosm; (B) carbon metabolism microcosm.

were filtered through 0.45 μ membrane filters. Each filter was then rinsed with sterile distilled deionized water (buffered, pH 7.2) to remove excess compound not utilized by the microbial biomass. Filters were dried and placed in scintillation vials containing 15 ml of phase-combining scintillant (Amersham).

All scintillation vials were counted for 10 min on a Beckman 250 liquid scintillator. A present error of 0.2% was used in conjunction with the external standard ratio mode for quench correction.

Data Analyses

Statistical methods used have included multivarient analyses to test the validity and reproducibility of the design features of the microcosm, together with SAS and Fortran programming approaches. Evaluations and tests of the variables considered, including population composition, enzyme response, and tagged compound turnover, have all been based on repeated runs of replicate sets.

RESULTS AND DISCUSSION

Enumeration of Total Heterotrophs and Chitinoclasts

Fluctuations in microbial biomass, expressed as CFU, in control and Guthion-fortified (10 ppm) microcosms are shown in Figs. 2 and 3. Data are given for chitinoclasts and heterotrophs under aerobic and anaerobic conditions. Statistical analysis showed significant variations in total heterotrophs and chitinoclasts ($P<0.01$). A rapid increase in microbial biomass occurred within the first 3 d of continuous application of the pesticide. After a 10-d application period, microbial concentrations returned to initial levels, with no significant change noted after the 10-d period.

Under anaerobic conditions, differences in the heterotrophic and chitinoclastic populations in the control and Guthion-fortified microcosm were evident. Significant increases attributable to azinphosmethyl application were noted under both aerobic and anaerobic conditions. Statistical analysis shows significant variations in total heterotrophs ($P<0.01$); however, no significant variation in chitinoclasts occurred after 5 d of continuous application of azinphosmethyl. Populations returned to initial levels, probably due to the containerization of the microcosm system, and remained invariant after 17 d.

Chitinoclasts comprised a significant portion of the aerobic microbial biomass prior to pesticide application and increased with continuous application of Guthion. A correlation between substrate turnover and enzyme activity with increased pesticide application also can be projected. It can be postulated that variations in the chitinoclastic group, as affected by Guthion, is indicative of the selectivity of pesticide interaction within the total microbial population. These and other data support the hypothesis that substrate turnover and specific enzyme activity are affected directly by application of Guthion. Elsewhere, Rajarum and Sethunathan (1975) reported correlations between presence of organic matter and degradation of the organophosphorus compound, parathion.

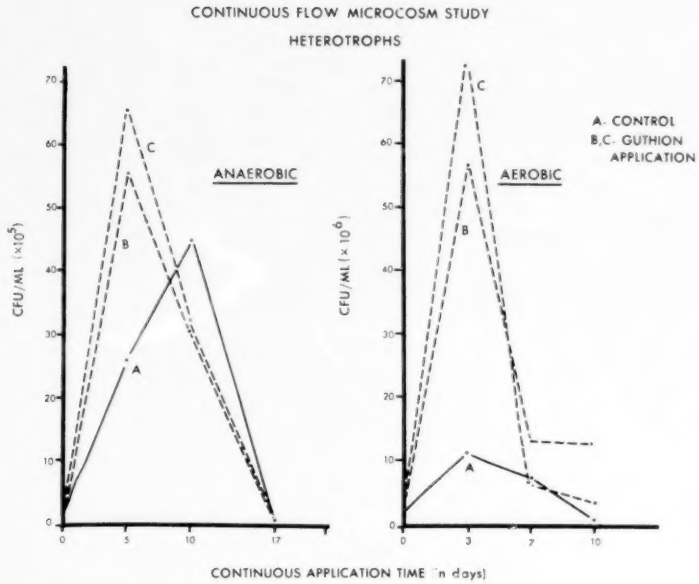


FIG. 2. Variations in total heterotrophs under aerobic and anaerobic conditions.

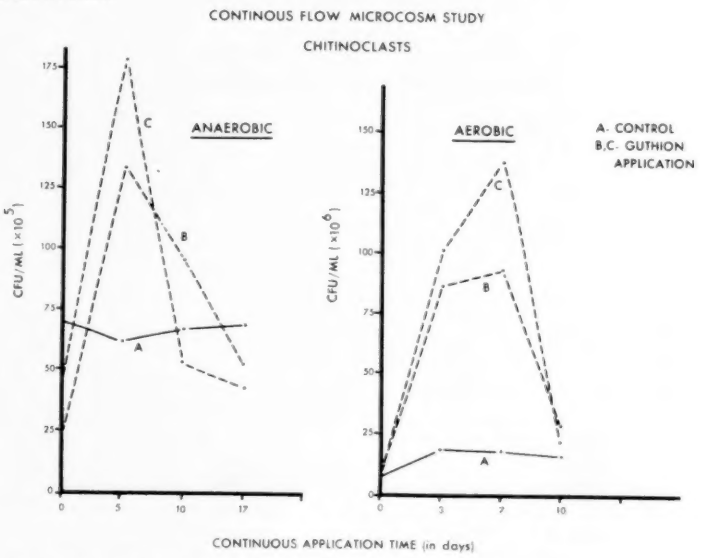


FIG. 3. Variations in total chitinoclasts under aerobic and anaerobic conditions.

Pesticide accumulation. The design of the continuous flow-through microcosm entails removal of an amount of fluid from the reaction vessel equivalent to that introduced. Preliminary tests in pesticide loss from the system due to flushing showed a depletion rate of 10%. At the prescribed rate of pesticide addition (0.105 ml/min), complete replacement of water within the microcosm with 10 ppm pesticide amended water would occur in 3.31 d. The detectable amount of pesticide after 3 d was considerably less. A significant accumulation of pesticide by the flooded soil occurred as a result of the continuous application, with high (>200 ppm) pesticide levels noted in sediment in both aerobic and anaerobic microcosms. This indicates that the sediment was an effective sink for the pesticide. It is noteworthy that the total microbial population under both aerobic and anaerobic conditions was not affected adversely by the extremely high concentrations of Guthion that occurred.

Carbon metabolism studies. The carbon-metabolism microcosm was designed to test for the rate of NAG utilization, as well as for relative respiration rates, by using the tagged compound, NAG (glucosamine-^{14}C). The objective was to obtain a more definitive picture of substrate utilization, i.e., NAG, as affected by pesticide treatment compared with earlier studies using chitin, and to determine more precise fluctuations in the microbial biomass due to pesticide addition based on $^{14}CO_2$ production. Earlier work, using the method of chitin assay described by Reissig et al. (1955), provided incomplete results on chitinase levels attributed to the heterogeneity of the population sample. Current efforts are being directed to modify the chitin assay broth to give reproducible results on substrate from flooded environments.

As noted in Fig. 4, both $^{14}CO_2$ respiration and ^{14}C-glucosamine

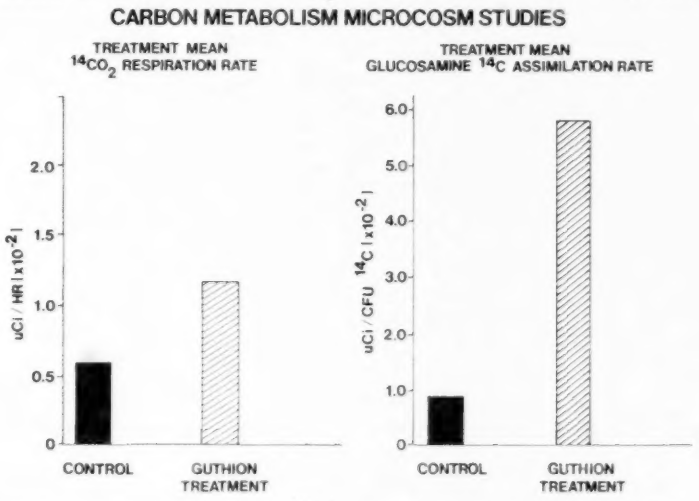


FIG. 4. Treatment variation in $^{14}CO_2$ respiration rate and glucosamine ^{14}C assimilation rate in control and pesticide-fortified microcosms.

rates increased with addition of Guthion. Variation in evolution of $^{14}CO_2$ with time by treatment proved to be highly significant ($P<0.01$). An immediate response to the tagged NAG was detected for static microcosms containing azinphosmethyl. A somewhat delayed response was noted for static microcosms without azinphosmethyl. Evolution of $^{14}CO_2$ for all microcosms leveled off after 48 h and remained invariant for a period of 24 d. The $^{14}CO_2$ evolved during these experiments accounted for between 27 to 42% of the added ^{14}C. Earlier, Bartha et al. (1967) reported organophosphates cause an initial increase in CO_2 production in upland soil, the magnitude of which is directly related to concentration applied.

Data suggest that Guthion has a positive and rapid action on NAG utilization, corresponding to increases in microbial activity concentrations and $^{14}CO_2$ evolution. This increase in microbial activity correlates to the response noted earlier in chitin-amended continuous flow-through microcosms. Thus, an enhancing effect by Guthion on chitin and its breakdown products is postulated. Chitin, an energy rich substrate readily colonized by the extant microbial community, provides an adsorptive medium for the pesticide. Elsewhere, Faust (1977), in his extensive review on organic pollutants in aquatic systems, noted the effect of organic matter content in controlling the extent of pesticide adsorption. Presence of Guthion has a positive effect on chitin utilization, which triggers a more complete and rapid mineralization of the xenobiotic.

Our field studies, to be reported elsewhere, support observations of other investigators on effect of organophosphate pesticides. Sethunathan (1973) noted a more rapid degradation of parathion in soils under flooded conditions compared with those under nonflooded conditions. Guthion is similar in action on the microbial community in flooded soils as are other organophosphates previously tested in upland, agricultural soils (Atlas et al. 1977). Rapid increases in bacterial concentrations coincide with rapid degradation of the pesticide. Higher pesticide concentrations correlate with higher bacterial concentrations. No significant variations in phosphatase and dehydrogenase activity are noted as a result of pesticide addition. These aspects, correlated with data from microcosm studies, are of significance in the selection and use of pesticides on upland, agricultural soils adjacent to highly productive estuarine systems.

Microbial and enzymatic correlations. Microbial and enzymatic response in control and pesticide-fortified microcosms over a 20-d period is given in Table 1. Azinphosmethyl was added to the test microcosm after 13 d. In general, enzyme activity was correlated with increases in microbial activity, with an order of magnitude change in fungal activity. Elsewhere, Bollag (1972) in his discussion on the biochemical transformation of pesticides by soil fungi, noted that bacteria and fungi will react to xenobiotic compounds differently; bacteria will often attach and degrade a compound to its mineral components whereas fungi will induce minor chemical changes. Considering the high fungal biomass found in both sugarcane and impounded swamp soils, significant degradation and modification of organophosphates such as Guthion by fungi may be a contributing factor in the relatively short half-life i.e., 7-7.5 d, of Guthion in soils. Over a 24-d test period, over 90

TABLE 1. Population variations and enzyme response for total heterotrophs in continuous flow-through microcosms

Microcosm	Day	Microbial Concentration (CFU) Filamentous Fungi (10^3)	Yeasts (10^3)	Bacteria (10^6)	Actinomycetes (10^6)	Phosphatase Activity mg/ml PNP[a]	Dehydrogenase Activity mg/ml TTF[b]
Control 1	0	28	3	11	2	0.270	0.016
	3	20	3	32	2	0.380	0.040
	7	27	2	40	7	0.360	0.044
	10	28	3	56	6	0.510	0.044
	13	33	3	57	6	0.350	0.038
	17	22	<1	12	3	0.410	0.031
	20	17	2	13	2	0.425	0.030
Control 2	0	38	12	11	2	0.300	0.017
	3	19	7	36	2	0.550	0.044
	7	27	3	52	8	0.558	0.046
	10	37	6	59	7	0.380	0.046
	13	37	6	51	7	0.425	0.043
	17	11	6	35	2	0.510	0.035
	20	9	4	19	2	0.400	0.025
Test 1	0	14	1	21	3	0.101	0.011
	3	9	3	29	2	0.275	0.042
	7	22	2	46	5	0.426	0.047
	10	32	2	57	5	0.371	0.046
	13*	33	2	56	4	0.395	0.048
	17	141	0	81	6	0.810	0.078
	20	62	0	54	2	0.545	0.059
Test 2	0	19	2	8	2	0.100	0.015
	3	10	0	32	3	0.380	0.030
	7	40	0	51	8	0.355	0.049
	10	71	0	52	4	0.430	0.048
	13*	68	0	63	4	0.425	0.049
	17	181	0	91	8	0.935	0.081
	20	121	0	67	7	0.570	0.062

*Guthion added.

[a]p-nitrophenol.

[b]2,3,5 triphenyltetrazolium formazan.

of the initial pesticide added was degraded to various breakdown products, possibly anthranilic acid and benzazimide (Liang and Lichtenstein 1972). This suggests that such products are not inhibitory to microbial respiration as discerned within the microcosm system.

The mean treatment variations for the various test parameters are given in Table 2. Analyses of data for the dependent variables, bac-

TABLE 2. Mean treatment variations and enzyme response for total heterotrophs in continuous flow-through microcosms

	Treatment I (Control)	Treatment II (Guthion)
Filamentous Fungi ($\times 10^3$)	24.5	58.8
Yeasts ($\times 10^3$)	4.3	2.0
Bacteria ($\times 10^6$)	34.5	50.6
Actinomycetes ($\times 10^6$)	4.1	5.1
Phosphatase (mg/ml PNP)*	0.416	0.437
Dehydrogenase (mg/ml TTF)**	0.036	0.048

* PNP = p-nitrophenol

**TTF = triphenyltetrazolium formazan

teria and fungi, showed significant variation between the control and test microcosms. Using analyses of variance procedures, significant($P < 0.01$) variation in biomass numbers was evident. The Duncan's multiple range test of variations in treatment means showed significant variations at an alpha level of 0.05. Significant variation in phosphatase level by respective treatment was not noted, in contrast to the significant variation in phosphatase activity observed over time of application. Highly significant variations ($P < 0.01$) in dehydrogenase activity were noted by treatment and time. Duncan's multiple range tests for dehydrogenase indicated significant variation between the control and experimental microcosms at an alpha level of 0.05. Both the phosphatase and dehydrogenase tests have been used by other investigators (Atlas et al. 1977; Casida 1977) in studying the effect of pesticides on soil microorganisms.

The type of information reported here, using a combination of microbial and enzymatic approaches, suggests validity of the bench-top microcosm approach as a valuable analytical tool for further studies of compound impact and substrate turnover. Rates of utilization and breakdown product generation can be monitored effectively. Certainly, there is a need to test any laboratory system in terms of "real world" phenomena. Current work is in progress on microcosm studies of parathion effect on chitin turnover.

The importance of monitoring chitinoclasts and chitin substrate cycling in terms of energy flow in estuarine food webs is clearly evident. Noteworthy *in situ* transformation rates (Hood and Meyers 1977b) indicate the significance of chitinolytic processes in early trophic level responses. The objective in these investigations has been to simulate the target environment under laboratory conditions rather than attempt to duplicate it. Nevertheless, if careful consideration is given to relevant physiochemical and microbial processes needed to simulate environmental conditions, pertinent information can be generated on the impact of toxic substances in aquatic systems using a model ecosystem or microcosm approach.

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